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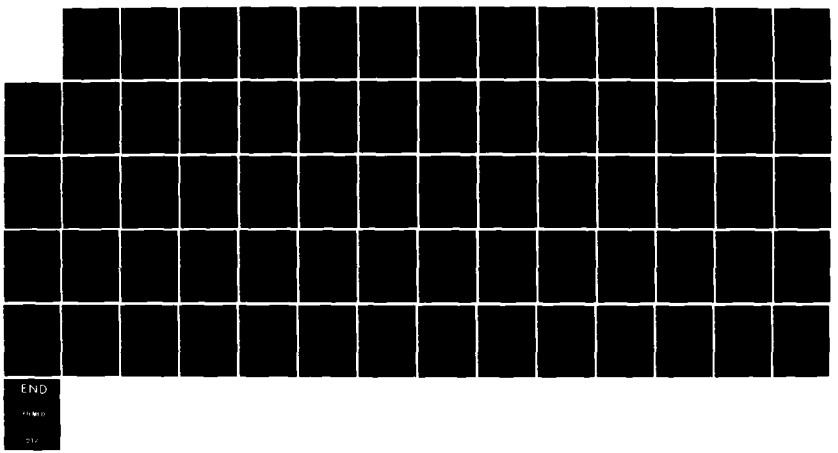

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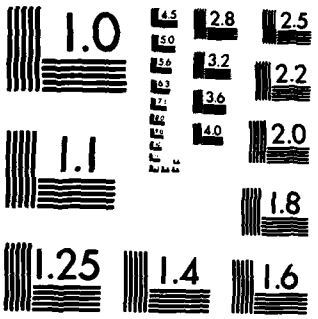

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Report No. 44-95-0183-TSI005

DEVELOPMENT OF SPECIAL BIOLOGICAL PRODUCTS (U)

Annual Progress Report

by

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Joseph L. DeMeio
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January 1983

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

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The Salk Institute
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19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Rift Valley Fever
Tissue Culture
FRhL-2
MRC-5
Venezuelan Equine Encephalomyelitis (VEE)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A. Tissue Culture:

Three production lots of FRhL-2, one each of MRC-5 and primary Duck and two lots of A. albopictus (C6-36) were stabilized and frozen. Eight lots of cells for use in testing were processed. A total of 5,148 amps of frozen cells were processed.

19. Continued
Western Equine Encephalitis (WEE)
Junin Virus
Korean Hemorrhagic Fever
Tularemia Vaccine
F. tularensis
Dengue Vaccine
Diagnostic Antiserum

20. Continued

B. VEE (C-84) Vaccine Development:
Potency testing on Lots C-84-6 and C-84-7 was completed. A second addendum covering VEE, inactivated, dried (TSI-GSD 205) vaccine lots C-84-2 through C-84-7 was prepared and transmitted to USAMRIID. Vaccine shipped to USAMRIID consisted of 75 vials (10 dose) C84-1A and 100 vials (10 dose) of C-84-3 Run 1.

C. WEE Vaccine Development:
Testing of WEE vaccine seed virus, strain CM4884 was completed. A test for adventitous agents in the seed was negative. The data on the vaccine was submitted as WEE vaccine, inactivated, dried, TSI-GSD 210.

D. Rift Valley Fever Vaccine Development:
The tests to certify the RVF virus, strain ZZ501, for vaccine production were completed. There are 100 x 1.0 ml vials of this seed virus stored at -65C.

E. Junin Virus Vaccine Development:
Safety tests on four fluids prepared at USAMRIID were conducted. Two freeze-drying runs using various suspending diluents were done.

F. Korean Hemorrhagic Fever
A total of 20 lots of 10 spot FA slides were produced and shipped. The 20 lots including one normal lot and 19 Hantaan virus infected lots, provided 5900 slides for use. One lot of two pools of rabbit anti Hantaan virus reference antisera was aliquoted in 0.2 ml amounts and freeze dried to provide approximately 200 vials for each pool.

G. Tularemia Vaccine Development:
Three batches of tularemia vaccine bacteria, strain LVS, were produced in a fermentor. The batches were separately concentrated by Pellicon ultrafiltration; the concentrates were pooled and diluted with an equal volume of sucrose-gel-agar for storage at -30C until lyophilized.

H. Dengue 1 Vaccine
Dengue 1 vaccine candidate seeds and Parent viruses were transferred from USAMRIID to TSI-GSD. Two passages of Strain 45AZ5 were made in certified FRhL-2 cells to prepare a Master Seed and a Production Seed. One passage of Strain TP79-56 was made in FRhL-2 to prepare a Production Seed. After testing and pooling, each production seed was passed once in certified FRhL-2 cells to prepare vaccine virus. The harvests were frozen at -70C in ten separate pools while testing was done. Strain 45AZ5 was thawed, pooled and freeze-dried in two runs. A monkey viremia study was done in addition to the required safety tests. Strain TP79-56 vaccine is still frozen in ten pools.

I. Diagnostic Antiserum Production
Anti-dengue virus mouse ascitic fluid and anti-bovine virus diarrhea rabbit sera were prepared.

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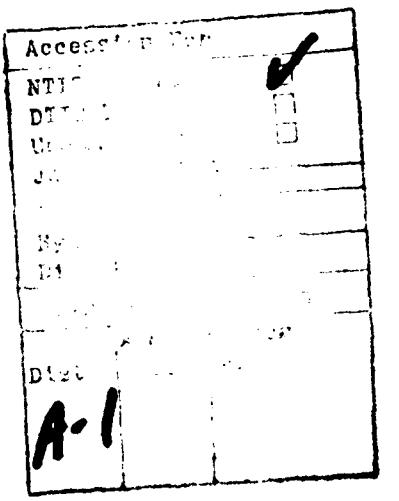
20. ABSTRACT (Continued)

J. Drug Screening Program

Plaque reduction tests were completed with RVF virus, five drugs; Pichinde virus, seven drugs; and JBE (Oct-541) virus, 20 drugs. Five drugs were tested by yield inhibition against Pichinde virus and peak yield harvest times were done against VEE, Pichinde and RVF viruses during the first quarter. Seventeen drugs were tested by plaque reduction for their effect on RVF, VEE, JBE and Pichinde viruses during the last quarter of 1982.



Summary

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Three production lots of FRhL-2, one each of MRC-5 and primary Duck and two lots of Aedes albopictus (C6-36) were stabilized and frozen this year. Eight lots of cells for use in testing were processed. A total of 5,148 amps of frozen cells were processed.

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D. Rift Valley Fever Vaccine Development

The tests to certify the RVF virus, strain ZZ501, for vaccine production were completed. These tests show that the virus fluid is free of bacterial and virus contaminants and contain only the RVF virus. There are 100 x 1.0 ml vials of this seed virus stored at -65C.

E. Junin Virus Vaccine Development

Safety tests on four fluids prepared at USAMRIID (Candidate #1 secondary seed and control fluid, Candidate #1 vaccine and control fluid) were conducted. Two freeze-drying runs using various suspending diluents were done. Assays for residual infectious virus were accomplished at USAMRIID.

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A total of 20 lots of 10 spot FA slides were produced and shipped this year. The 20 lots including one normal lot and 19 Hantaan virus infected lots, provided 5900 slides for use.

One lot of two pools of rabbit anti Hantaan virus reference antisera prepared last year was aliquoted in 0.2 ml amounts and freeze dried to provide approximately 200 vials for each pool.

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Anti-dengue virus mouse ascitic fluid and anti-bovine virus diarrhea rabbit sera were prepared. These stock reagents are stored at -65C and will be tested for use.

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Plaque reduction tests were completed with RVF virus, five drugs; Pichinde virus, seven drugs; and JBE (Oct-541) virus, 20 drugs. Five drugs were tested by yield inhibition against Pichinde virus and peak yield harvest times were done against VEE, Pichinde and RVF viruses during the first quarter of 1982.

Seventeen drugs were tested by plaque reduction for their effect on RVF, VEE, JBE and Pichinde viruses during the last quarter of 1982.

Foreword

The authorization for the work contained herein was authorized under Contract No. DAMD17-78-C-8018, titled, "Development of Special Biological Products".

This annual report covers the period of January 1, 1982 through December 31, 1982. In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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Tissue Culture

I. Introduction

Three production lots of FRhL-2, one each of MRC-5 and primary Duck and two lots of Aedes albopictus (C6-36) were stabilized and frozen this year. Eight lots of cells for use in testing were processed. A total of 5,148 amps of frozen cells were processed.

II Process Studies

A. Production Cells

1. FRhL-2 Diploid Cell Line

Three production lots of FRhL-2, Lots 28, 29 and 30, were prepared and certified as summarized in Table 1. The tumorigenicity test remains to be done on Lot 30.

A total of 402 amps of FRhL-2 was used for Dengue vaccine and testing procedures.

2. Primary Chick Embryo Cells

Small batches were processed for safety tests.

3. MRC-5 Diploid Cell Line

Lot 15 MRC-5 was the first lot of cells prepared from the new production seed PS2. It was harvested at P19 and all testing was satisfactory as shown in Table 2. A full karyologic analysis was done and is summarized in Table 3. Three hundred ampules of this lot were shipped to USAMARIID prior to completion of testing. Additionally, 50 ampules of lot 9 were shipped.

A total of 44 ampules were used for testing.

4. Aedes albopictus (C6/36)

Chromosome analyses were completed on the two production Seeds 1 and 2, this year. Table 4 summarizes this data indicating that both seeds were stable after two to six passages and comparable to the initial study on Passage 11 cells.

Testing on three production lots of Aedes albopictus, Lots 1, 2 and 3 is shown in Table 5. Production seed 1 was used to prepare Lot 1, and PS2 was used for Lots 2 and 3. Lot 3 was grown in suspension culture. A single idiogram monitor test was done on each of the three lots. The

tumorigenicity test remains to be done on Lot 3. Some adaptation to suspension culture was indicated when these cells were placed in monolayer as indicated by the increased time needed for sheeting.

Shipment of 168 ampules of Lot 2 was made to WRAIR. Another 114 amps of all lots were used for testing.

5. Primary Duck Cells

One lot of primary duck cells (PD3) was prepared from SPAFAS duck eggs (13 day). A total of 369 amps, each containing 134 x 10 cells at 74 percent viability was produced. Testing is shown in Table 6.

6. Primary Dog Kidney (Dow)

A total of 28 ampules from dogs #152 and #158 were shipped to Dr. Halstead in Hawaii.

B. Test Cells

1. LLC-MK2

Five lots of cells were frozen at different passage levels. Two of these lots were adapted to growth at 39° C prior to freeze down (P 323 and 332). These cells (39° temp. variants) grow at a rate that is twice that observed at 37°C and were intended for use in ts studies with Dengue. However, all lots were contaminated with Mycoplasma. A new start from ATCC is planned.

Attempts to grow these variants at 41 C met with failure.

2. Vero

Two lots of Vero, one from Merieux Institute, France (French) and Clone 76 (ATCC) were processed this year. Both are free of Mycoplasma. They are included in the year-end inventory.

3. Miscellaneous

One small lot of BHK-21 was placed in inventory. A total of 1197 ampules of test cells were processed this year.

III Experimental

A. Microcarrier

Some success in growing MRC-5 in a 5 liter (Techne) fermentor with Cytodex III was achieved. Vero Clone 008 was also grown on Cytodex III in a 500 ml volume. FRhL-2 has not, as

yet, been successfully cultured. However, the technique for MRC-5 culturing has to be tried yet on FRhL-2 cells since modification may be beneficial. A comparison of the growth of these cells is given in Table 7. Best efficiency is shown with Vero, followed closely by MRC-5. FRhL-2 failed to grow after 2 days indicating CO_2/O_2 tensions were not ideal. We will have to include gas monitoring in our culture conditions. Other points that appear to help in culturing diploids are as follows:

1. Best speed about 16-18 rpm.

2. Dropping the initial 20% fetal bovine serum to 10% should be in gradual steps. Media changes should be gradual - perfusion probably best.

3. Checking and adjusting the pH should be automatic.

B. Serum

Nu-serum, a semi-synthetic from Collaborative Research was evaluated with BHK-21 cells. Cells grew and sheeted the same as with fetal bovine serum but cells did not maintain past 8 days when held indicating some deficiency.

C. Primary Cells for Safety Tests

1. Primary green monkey kidney was obtained from WRAIR for use in safety testing Junin and Dengue 1 vaccines. After 1 passage, 9 amps were frozen and after 2 passages, 44 amps (3.4×10^6 cells/85% viable).

2. Primary rabbit kidney, obtained from Flow Labs, was used for safety testing of Junin and Dengue 1 vaccines. Primary was frozen in 11 amps, Pass 1 in 38 amps (10.6×10^9 /98% viable) and Pass 2 in 11 amps.

IV New Tissue Culture Wing

The foundation, footings and initial grading for the tissue culture wing were finished.

V Cell Inventory and Use - 1982

The inventory is given as Table 8, showing our current stocks. A total of 546 amps were shipped and 741 were used here for testing and Dengue Vaccine purposes. A total of 3951 amps for vaccine work and 1197 amps for testing were produced this year.

VI Conclusion

The training of personnel kept contamination at an almost non-existent level this past year with the exception of the LLC-MK2 cells which were, in all probability, contaminated when

they were received. This brought to our attention the need to quarantine new cell starts until they have been fully evaluated. The on-coming new wing will allow us space for this purpose.

Table 1

Certification of Three Lots of FRhL-2 (P16)

Item	Results Lot 28	Lot 29	Lot 30
No. bottles harvested	719	527	719
Surface area (cm^2)	107,850	79,050[1]	107,850
Total cells ($\times 10^{10}$)	0.819	0.958	1.62
Cells/cm ($\times 10^5$)	0.759	1.21	1.50
No. amps	433	452	629
Cells/amp ($\times 10^6$)	20	24	25.8
Viability (%)	90	90	94
Sheetability -- 1 amp - 10 x 75 cm^2	3-4 days	3 days	3 days
1 amp - 10 x 150 cm^2	5 days	4 days	5 days
Bulk sterility	Sterile	Sterile	Sterile
2 week-hold of 1-2% cell sample after harvest	Normal	Normal	Normal
30-day hold of harvest fluids	Sterile	Sterile	Sterile
PPLO	Negative	Negative	Negative
Hemadsorption: cells from sheetability	Negative	Negative	Negative
2 week-hold-harvest cells	Negative	Negative	Negative
M. tuberculosis (Lowenstein-Jensen)	Negative	Negative	Negative
Tissue culture safety -- a) MRC-5	Passes	Passes	Passes
b) FRhL-2	Passes	Passes	Passes
c) RK-13	Passes	Passes	Passes
d) Vero & subpass	Passes	Passes	Passes
e) CV-1 & subpass	Passes	Passes	Passes
f) CEC	Passes	Passes	Passes
Egg safety (allantoic)	Passes	Passes	Passes
Tumorigenicity (newborn hamster/ALS	Negative	Negative	ND[2]
Karyology	Normal[3] diploid	Normal[3] diploid	Normal[3] diploid

1 Broken flask of final suspension caused reduction.
2 ND = not done
3 Single idiogram monitor test.

Table 2

Preparation and Testing of MRC-5 Lot 15 (P19)

Item	Result
No. bottles harvested	618
Surface area (cm^2)	92,700
Total cells ($\times 10^{10}$)	1.06
Cells/cm^2 ($\times 10^5$)	1.14
No.amps	422
Cells/amp ($\times 10^6$)	25.2
Viability (%)	93
Sheetability -- 1 amp - 10 x 75 cm^2	3 days
1 amp - 10 x 150 cm^2	4 days
1 amp - 850 cm^2 roller	3 days
Bulk sterility	Sterile
2 week-hold of 1-2% cell sample after harvest	Normal
30-day hold of harvest fluids	Sterile
PPLO	Negative
Hemadsorption: cells from sheetability	Negative
2 week-hold-harvest cells	Negative
M. tuberculosis (Lowenstein-Jensen)	Negative
Tissue culture safety -- a) French Vero	Passes
b) CV-1	Passes
c) RK-13	Passes
d) FRhL-2	Passes
e) MRC-5	Passes
f) CEC	Passes
Egg safety (allantonic)	Passes
Tumorigenicity (newborn hamster/ALS)	Passes
Karyology	Normal diploid

Table 3

Chromosome Analysis of MRC-5 Lot 15

TOTAL NO. OF CELLS COUNTED 400

MODAL CHROMOSOME NO. 46

NO. OF CHROMOSOMES	44	45	46
NO. OF CELLS	9	37	354

POLYPLOIDY - 2.2%
TOTAL BREAKS - 11
TOTAL GAPS - 15
PERCENTAGE OF THE CELLS WITH BREAKS AND/OR GAPS - 6.3%

88.5% of the cells observed showed 2n = 46 while 11.5% were hypodiploid. The incidence of polyploid was 2.2% on the basis of 500-cell count. The incidence of the cells with breaks and/or gaps was 6.3% All of the above incidences fall within the respective upper acceptable limits at 95% confidence. This, together with the analysis of 20 representative idiograms, suggest that MRC-5, Lot 15 is a normal diploid cell line.

September 24, 1982

Table 4

Chromosome Analysis of A. albopictus (C6/36)

	Previous (3/80)	Prod.Seed #1 (P11)		Prod.Seed #2 (P12)	
	P11 (Comparison)	P14	P17	P14	P16
Total no. of cells counted	100	100	100	100	100
Modal chromosome no.	6	6	6	6	6
No. cells with					
Breaks	19	18	13	17	16
Gaps	19	8	11	10	8
Other abnormalities	1	2	3	0	2
Secondary constriction - like images	4	18	16	12	13
% cells with aberrations	38%	37%	36%	38%	35%
Polyploidy	13%	11.2%	9.0%	8.0%	8.2%

No. of chromosomes		4	5	6	7	8	9	10	11	12	18
P.S.#1 No. of cells	P14	4	2	84	2	1	1	2	2	1	1
	P17	3	11	81	1			1		3	
P.S.#2 No. of cells	P14	4	2	82					2	9	1
	P16	3	3	88				1	1	4	
MARCH 21, 1980	P11		2	82	1			1	1	13	

The above data indicate that the passages do not make any significant differences in the karyological parameters checked. And these data are also well in agreement with those observed on P11 of A. albopictus C6/36 except polyploidy. The higher incidence of polyploidy of P11 cells is considered to be due to the prolonged treatment of colcemid. All of these observations together with 20 representative idiograms indicate that A. albopictus C6/36 is a stable cell line at the passages examined.

Table 5

Preparation and Testing A. albopictus (C6/36)

Item	Lot 1(P15)[1]	Lot 2(P14)[2]	Lot 3(susp.)[3]
No. bottles harvested	350	428	-
Surface area (cm^2)	52,500	65,700	-
Total cells ($\times 10^{10}$)	0.6	3.3	3.63
Cells/cm^2 ($\times 10^5$)	1.14	5.02	9.95
No. amps	444	551	1140
Cells/amp ($\times 10^6$)	13.6	60	31.8
Viability (%)	71.5	83	76
Sheetability - 1 amp - 10 x 75 cm^2	6 days	3 days	8 days
1 amp - 10 x 150 cm^2	7 days	4-5 days	9-10 days
Bulk sterility	Sterile	Sterile	Sterile
2 week-hold of 1-2% cell sample after harvest	Normal	Normal	N.A.[4]
30-day hold of harvest fluids	Sterile	Sterile	Sterile
PPLO	Negative	Negative	Negative
Hemadsorption: cells from sheetability	Negative	Negative	Negative
2 week-hold-harvest cells	Negative	Negative	N.A.
M. tuberculosis (Lowenstein-Jensen)	Negative	Negative	Negative
Tissue culture safety - a) Vero CL 008 & subpass	Passes	Passes	Passes
b) CV-1 & subpass	Passes	Passes	Passes
c) RK-13	Passes	Passes	Passes
d) FRhL-2	Passes	Passes	Passes
e) MRC-5	Passes	Passes	Passes
f) CEC	Passes	Passes	Passes
Egg safety (allantoic)	Passes	Passes	Passes
Tumorigenicity (newborn hamster/ALS)	Passes	Passes	N.D.
Karyology	Satisfactory[5]	Satisfactory[5]	Satisfactory[5]

1/ From PS 1
2/ From PS 2
3/ From PS 2 - Suspension Culture of 36,500 ml
4/ N.A. = not applicable, N.D. = not done

5/ Single idiogram monitor test.

Table 6

Preparation and Testing of Primary Duck Embryo Cultures Lot 3

Item	Result
No. embryos harvested	482
Total cells (x 10^{10})	4.9
No. Amps	369
Cells/amp (x 10^6)	134
Viability (%)	74
Sheetability - 1 amp - 5 x 75 cm^2	6 days
1 amp - 1 x 150 cm^2	5 days
Bulk sterility	Sterile
PPLO	Negative
Hemadsorption: cells from sheetability	Negative
M. Tuberculosis (Lowenstein-Jensen)	Negative
Tissue culture safety - a) Vero & subpass	Passes
b) CV-1 & subpass	Passes
c) RK-13	Passes
d) FRhL-2	Passes
e) MRC-5	Passes

Table 7

Growth of Three Cells In Five Liter
Microcarrier Culture
of Cytodex III

	CELL		
	Vero (008)[1]	MRC-5 P17	FRhL-2 P15
Cytodex III weight	15 g	10 g	10 g
No. microcarriers	60×10^6	40×10^6	40×10^6
Cells inoculated/bead	10	20	20
No. cells/bead day 1	9	7	18
Attachment efficiency	90%	35%	90%
Days to peak growth	8	5	2
No. cells/bead at peak[2]	129	60	38
Theoretical no. cells/bead	154	76	76
Microcarrier surface sheeted	84%	79%	50%
Increase in no. cells/(fold)[3]	14.3	8.6	2.1

1) Adjusted to 5L culture from 500 ml culture size for comparison.
2) Cells either remained at this level for 2 - 4 days or started to decrease in number and viability.
3) No. cells/bead at peak/no. cells per bead day 1.

Table 8

Cell Inventory and Use-1982

Item No	Cell	Lot No.*	Pass	Date Frozen	Cell Count Per Amp (x 10^6)	Viability (%)	No.-Jan 81	Ampoules Shipped	Ampoules Used	Current Inventory	Use
1	FRhL-2	PS	10	2/14/78	6.4	94-98	79		5	74	Certified Cells for Vaccine Preparation
		8 OPS	17	11/23/76	8.0	75	284			284	
		14	17	02/22/78	26.0	96-98	288		4	284	
		18	16	09/20/78	31.2	95-100	14			14	
		21	16	03/20/79	29.8	93	297		184	113	
		24	16	04/02/80	31.4	90	449		1	448	
		26	16	02/10/81	26.0	93	498		3	495	
		27	16	02/04/81	16.0	91	728		2	726	
		28	16	03/24/82	20.0	90	-		56	387	
		29	16	03/30/82	24.0	90			65	387	
		30	16	05/18/82	26.0	94			82	547	
2	FCL	Seed	7				1			1	
		OPS	16	05/17/77	5.4	90-94	L93			193	
3	IMR-90		7	05/03/77			3			3	
			8	05/06/77			3			3	
			9	05/11/77			3			3	
		MS	10	05/16/77	4.9	94	180			180	
		PS	14	06/01/77	3.4	99	45			45	
		1	21	11/14/77	37.7	94-97	301			301	
4	MRC-5	PS-2	14	12/28/81	12.0	94	124		11	113	
		9	23	06/10/80	21.3	98	193	50		143	
		10	23	07/15/80	23.6	98	376			376	
		12	23	11/04/80	20.6	92	607			607	
		15	19	01/20/82	25.2	93		300	33	89	
5	Primary Duck	3	Prim	01/26/82	134.0	74			55	314	
6	IMR-91		8	08/25/78	1.1	91	3				
		MS	10	08/31/78	5.2	100	98		1	97	
7	Dog Kidney	Dow	Prim	04/05/77	(Rec'd)		1375	28		1347	
A. 8	albopictus (C6/36)	PS1	11	07/27/81			89		3	86	
		1	15	12/15/81	13.6	71	436		32	404	
		PS2	12	12/17/81	11.2	65	93		5	88	
		2	14	01/28/82	60.0	83		168	47	336	
		3	Susp	02/05/82	31.8	76			27	1113	

Cell Inventory - continued

Item No	Cell	Lot No.*	Pass	Date Frozen	Cell Count Per Amp (x 10)	Viability (%)	No.-Jan 81	Ampoules Shipped	Ampoules Used	Current Inventory	Use
9	BSC-1		76	02/14/75	14.0	84-87	19			19	
10	CV-1		29	12/21/76	1.3	85	10			10	
			36	10/20/78			68		1	67	
11	KB			08/18/75	14.0	89-98	45		1	44	
12	LLC-MK2		264	02/11/75	4.0	78	31		2	29	
			270	05/28/82	11.5	94			34	108	
			317	07/01/82	9.1	98			13	279	
			328	08/20/82	9.8	94			2	177	
		Temp. VAR.(39 C)	323	09/17/82	6.6	93			6	44	
		Temp. Var.(39 C)	332	09/21/82	6.4	94			6	70	
13	RK-13		73	06/16/75	9.0	83	35		4	31	
14	Vero		122	04/24/75	2.0	82	34		2	32	
			137	08/30/79	32.4	96	81		7	74	
		Clone 008	26	11/12/81	10.0	93	370		12	358	
		French	157	02/16/82	10.5	82			9	105	
		Clone 76	32	06/15/82	13.5	90			12	224	
15	BHK-21		57	05/14/79(Recd)			5		1	4	
		1	58	06/12/79			18		4	14	
		2	57	11/04/82	22.8	68				108	
16	L929		556	12/18/79(Recd)			1			1	
			559	01/10/80			87		1	86	
17	CER-4	1	17	03/30/81	25.7	99	71			71	
18	PK(15)	1	136	06/24/81	25.6	97	101		1	100	
19	Bovine turbinate	1	24	07/31/81	6.2	96	104		6	98	
20	A549	1	81	10/02/81	8.3	97	140		1	139	

Test Cells

VEE (C-84) Vaccine Development

I. Introduction

Potency testing on Lots C-84-6 and C-84-7 was completed this year. A second addendum covering VEE, inactivated, dried (TSI-GSD-205) vaccine Lots C-84-2 through C-84-7 was prepared and transmitted to USAMRIID. Vaccine shipped to USAMRIID consisted of 75 vials (10 doses) of C84-1A and 100 vials (10 doses) of C-84-3, Run 1.

II. Vaccine Testing

Lots C-84-6 (4 runs), C-84-7 (3 runs), C84-1 and C84-1A were tested for potency in guinea pigs to complete testing. Those data are given in Table 9. As can be seen, all ED_{50} values are similar except for Lot C-84-1A which required twice the volume to immunize 50 percent of the guinea pigs against challenge with Trinidad Strain virus.

A total of 75 vials of C-84-1A vaccine and 100 vials of C-84-3, Run 1 vaccine were shipped to USAMRIID.

A second addendum for TSI-GSD-205 covering Lots C-84-2 through C-84-7 was transmitted to USAMRIID.

III. Vaccine Virus Preparation

Approximately 2 liters of VEE, C-84 virus was prepared in rolling cultures of CEC using C-83 virus seed following the production protocol. The harvested material was filtered and frozen at -65°C. It was shown to be attenuated by suckling mouse inoculation and free of bacterial contamination. The titer was $10^{9.8}$ /ml in CEC cells. A total of 1.5 liters of this material was shipped. A separate report on preparation and testing was also sent.

Table 9

VEE Vaccine Potency Testing
(TSI-GSD 205)

VEE Vaccine		Serological Conversions by HI from Sera of Guinea Pigs Given Vaccines Diluted 1:5	1:25	ED_{50} (ml) Probit
Lot C-84-6	Run 1	90%	60%	0.005
	Run 2	90%	40%	0.006
	Run 3	90%	30%	0.007
	Run 4	89%	30%	0.005
Lot C-84-7	Run 1			0.006
	Run 2	70%	30%	0.007*
	Run 3	80%	20%	0.008*
Lot C-84-1		90%	50%	0.005
Lot C-84-1A		100%	0%	0.0125

* Mean of two or more tests.

WEE Vaccine Development

I. Introduction

Testing of WEE vaccine seed virus, strain CM4884 was completed this year. A test for adventitious agents in the seed was negative.

The data on the vaccine was submitted as WEE vaccine, inactivated, dried, TSI-GSD 210.

II. Process Studies

A summary showing the passage history and testing of WEE vaccine seed virus, strain CM4884 is given as Table 10

An adventitious agent test in tissue culture was devised and is shown in Table 11. Antisera for the test was prepared in rabbits injected twice (0.5 ml 0 and 14 days) i.m. with a mouse brain suspension of B-11 strain WEE. The test was performed using unclarified virus from WEE vaccine preparation, Lot 1-81 ($10^{8.8}$) since it was impossible to neutralize the production seed (20% embryo slurry) having a titer of $10^{-10.5}$. This virus was diluted in undilute antisera (0.1 ml virus, 0.9 ml antisera) from 10^{-1} through 10^{-11}. Controls were prepared using fetal calf serum for dilution in a like manner.

After neutralization (overnight at 4°C and 1 1/4 hrs at 37°C), each dilution was inoculated onto four 25 cm^2 flasks (0.2 ml each) of MRC-5 cells and incubated at 35°C.

Twenty-four hours later, 0.5 ml aliquots were removed from each culture and pooled (2 ml/diln.). These samples were held frozen at -65°C for passage on MRC-5, CEC and A. albopictus cells (Step 6 in Flow diagram). Observation of the MRC-5 cultures for 15 days is shown in Table 12. No CPE was observed and the test cells were negative to hemadsorption with guinea pig red blood cells after 15 days.

A summary of the observations of 24 hour aliquots passed in MRC-5, CEC and A. albopictus cells is given as Table 13. No CPE was observed in MRC-5 while the 10^{-1} virus antisera mixtures showed CPE in CEC and A. albopictus cells. An aliquot of the 10^{-1} culture fluid from A. albopictus, reneutralized with WEE B-11 antisera indicated the CPE to be caused by WEE virus as shown in Table 14.

The observed data are consistent with the conclusion that no adventitious agents are present in WEE vaccine seed virus, strain CM4884 under the conditions of the testing.

A submission on WEE, inactivated, dried, TSI-GSD 210 was transmitted to USAMARIID.

Table 10

Passage History and Testing of WEE Vaccine
Seed Virus, Strain CM4884

Passage	Passed in	By
Isolate	Mosquito isolate - squash	Fort Collins
1	Certified CEC (pool 28)	USAMRIID
2	Certified CEC-SPAFAS MR63	TSI
3	SPAFAS Chicken Eggs-8 day LEB 6- 20% homogenate	TSI (Production Seed)

Test		Result
Sterility:	Bacterial	Sterile
	TB cultural	Negative
	PPLO	Negative
Identity:	Direct- B-11 antiserum-Vero TC	LNI 2.0
	Indirect[1] - HAI immunized g.p. sera	Satisfactory
	- Challenge immuinized g.p.	Satisfactory
Titer:	$TCID_{50}$ log_{10}/ml (20% embryo suspension)	10.5
Adventitious agent[2]		Satisfactory

1] Guinea pigs immunized with WEE Lot 1-81 vaccine prepared with this seed virus.

2] This report.

Table 11

Flow Diagram
WEE Adventitious Agent Test

Step	Procedure
1	WEE virus diluted in antiserum 10^{-1} - 10^{-11}
	(Virus control diluted in normal fetal calf serum)
2.	20 hrs 5°C
	1.25 hrs 37°C
3.	Each dilution inoculated into MRC-5 cell cultures
4.	24 hrs: Pool of fluid from each dilution
5.	MRC-5 cultures observed 15 days. HAd test. (Table 12)
6.	Fluid from each dilution inoculated (subpassed) into:

MRC-5 cells (Table 13) | Chick embryo cells (Table 13) | A. albopictus cells (Table 13)

7-day fluid 10 cultures titrated in CEC (Table 14)

Table 12

Observations-WEE Virus Adventitious Agent Test:
Virus-Serum Inoculation on MRC-5 Cells

	Observation (Days)											
	Virus + Antiserum						Virus + FCS					
Diln.	1	3	6	9	12	15[2]	1	3	6	9	12	15
	(CPE/total)											
10^{-1}	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	4/4	-	-	-
10^{-2}	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	4/4	-	-	-
10^{-3}	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	2/4	3/4	3/4	3/4
10^{-4}	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	1/4	1/4	1/4	1/4[3]
10^{-5} [1]	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Cell Controls	0/2	0/2	0/2	0/2	0/2	0/2						

1] Dilutions 10^{-6} - 10^{-11} observed with no CPE occurring.

2] HAD test done with guinea pig redblood cells was negative. The 10^{-1} through 10^{-4} cultures were tested.

3] Titer $10^{-5.5}$ /ml vs $10^{-8.8}$ /ml of untreated virus.

Table 13

Observations-WEE Adventitious Agent Test: Subpassage of 24-hr Fluid from Serum-Virus Cultures

MRC-5

Virus-Antiserum Sample Dilution	Observation (days)					
	1	3	6	9	12	15
	(CPE/total)					
10^{-1}	0/2	0/2	0/2	0/2	0/2	0/2
10^{-2}	0/2	0/2	0/2	0/2	0/2	0/2
10^{-3}	0/2	0/2	0/2	0/2	0/2	0/2
10^{-4}	0/2	0/2	0/2	0/2	0/2	0/2
10^{-5}	0/2	0/2	0/2	0/2	0/2	0/2

CEC

	Observations (days)		
	1	4	7
	(CPE/total)		
10^{-1}	0/2	1/2	1/2
10^{-2}	0/2	0/2	0/2
10^{-3}	0/2	0/2	0/2
10^{-4}	0/2	0/2	0/2
10^{-5}	0/2	0/2	0/2

A.albopictus (C6/36)

	Observations (days)					
	1	3	6	9	12	15
	(CPE/total)					
10^{-1}	0/2	0/2	1/2*	1/2	2/2	2/2
10^{-2}	0/2	0/2	0/2	0/2	1/2	2/2
10^{-3}	0/3	0/2	0/2	0/2	0/2	0/2
10^{-4}	0/2	0/2	0/2	0/2	0/2	0/2
10^{-5}	0/2	0/2	0/2	0/2	0/2	0/2

* Aliquot reneutralized and passed in CEC (Table 14).

Table 14

Observations on WEE Adventitious Agent Test:
Titration of Fluid from 7-day 10^{-1} Culture
*A. albopictus** on CEC

Dilution	Observation (days)					
	FCS Diluent			Antiserum Diluent		
	1	3	7	1	3	7
	(CPE/total)					
10^{-1}	2/2	-	-	0/2	0/2	0/2
10^{-2}	0/2	0/2	0/2	0/2	0/2	0/2
10^{-3}	0/2	0/2	0/2	0/2	0/2	0/2
10^{-4}	0/2	0/2	0/2			
10^{-5}	0/2	0/2	0/2			

*Reneutralized with WEE B-11 antiserum. Controls with fetal calf serum, 37°C/1 hour with agitation.

Rift Valley Fever Vaccine Development

I. Introduction

The tests to certify the RVF virus, strain ZZ501, for vaccine production were completed. These tests show that the virus fluid is free of bacterial and virus contaminants and contain only the RVF virus. There are 100 x 1.0 ml vials of this seed virus stored at -65C.

II. Certification Test Results

A summary of the passage history and certification results are shown in Table 15. Table 16 gives the results of tests for adventitious agents.

Table 15

Passage History and Testing of RVF Virus
ZZ501 Vaccine Seed Virus

Passage History

Passage	Virus Source	Passage Host	Where
1	Human Sera (Egypt)	FRhL-2 Cells	USAMRIID
2 (Seed)	Pass 1	FRHL-2 Cells	TSI-GSD

Certification Status

Test		Result
Sterility:	Bacterial	Satisfactory
	TB Cultural	Satisfactory
	PPLO	Satisfactory
Control cell observation (from seed prep.)		Cells normal (18-days post harvest)
Adventitious agent		Passes*
Identity		Satisfactory (LNI 3.2)
Titer: $TCID_{50}$ log_{10} /ml	Vero cells	7.6
	BHK-21 cells	8.3
Hemagglutination		1:64

*See Table 16, this report.

Identity

Virus Strain	Virus Titer Entebbe Atiserum/Virus (LD_{50} log_{10})	Normal FCS/Virus	LNI
ZZ501 Seed	3.5	6.7	3.2
Entebbe Control	2.5	5.5	3.0

Table 16

Tissue Culture Adventitious Agent Procedure for RVF Virus, Strain ZZ501 Vaccine Seed

Step	Procedure	Result
1.	a. RVF virus dilutions (10^{-1} - 10^{-9})* Neutralized with RVF Entebbe antisera (0.9 ml antisera, 0.1 ml virus). Inoculated 0.2 ml onto each of 4 x 25 cm^2 BHK-21 cultures/ dilution. Incubated at 35°C	No. CPE/7 days HAd negative
	b. Virus control - virus 0.1 ml plus 0.9 ml fetal calf serum	$10^{6.3}$
2.	48 hour samples of each dilution* (step 1)	
	a. Passed in CEC (2 x 25 cm^2 each)	1/2 cultures CPE at 10^{-1} diln. 0/2 10^{-2} through 10^{-5}
	b. Passed in *Aedes albopictus* cells	2/2* cultures CPE at 10^{-1} through 10^{-3} 1/2 CPE at 10^{-4} . 0/2 10^{-5} through 10^{-9}
3.	*Fluid from 10^{-1} *A. albopictus* cultures (step 2b)	Control cultures CPE at 10^{-1} and 10^{-2}
	Reneutralized with RVF Entebee antisera and titered on CEC	Neutralized-no CPE at 10^{-1}-10^{-3}

Junin Virus Vaccine Development

I. Introduction

Safety tests on four fluids prepared at USAMRIID (Candidate #1 secondary seed and control fluid, Candidate #1 vaccine and control fluid) were conducted. Two freeze-drying runs using various suspending diluents were done. Assays for residual infectious virus were accomplished at USAMRIID.

II. Safety Tests

Safety tests on four fluids prepared at USAMRIID (Candidate #1 secondary seed and control fluid, Candidate #1 vaccine and control fluid) were done in rabbits, adult mice and cell cultures (FRhL-2. Primary GMK, Primary rabbit kidney and MRC-5). Microbial sterility including Mycoplasma testing were also conducted. A problem developed with the secondary seed control fluid in that it was bacterially contaminated. The probable cause was the leaking of the flask-container in which it was shipped. A second test sample was also contaminated. This material was from a 2nd harvest dated 19 April 1982. The organism was a gram negative rod, slightly branched and tentatively identified at Pocono Hospital as Acinetobacter lwoffe. Secondary seed control fluid from the first harvest dated 9 April 1982 was tested and passed.

A summary of test results is given in Table 17.

In the tissue culture safety test in MRC-5, it is interesting to note that Junin virus causes delayed CPE in MRC-5 cells. This may be of benefit in future growth or assay of the virus. The antisera used (Rhesus T-23) did not completely neutralize the virus in this test. A repeat test is scheduled.

III. Freeze-drying

Two freeze-drying trials were made with Junin Candidate #1, P2, 19 March 1982 (Mfg.date). Seven media were evaluated in the first run and two were selected for the second trial. See Tables 18 and 19. Virus was diluted 1:30 for drying and all viral titrations were done at USAMRIID.

In trial 1, the titer of the pool (frozen/thawed) was 2 logs lower than the original titer of this virus (original titer was 1.8×10^6 PFU/ml and virus pool was 1.2×10^4 PFU/ml). The cause of this is unknown. The theoretical titer of the virus pool (calculated) if diluted 1:30 would be 3.9×10^2 PFU/ml. A mean titer of all diluted, frozen/thawed samples was 5×10^2, indicating no loss during these procedures. After drying, it is evident from the percentage recovery column that Media 5 (water-based with degraded gelatin and sorbitol) was superior in recovery to the remaining diluents, showing no drying loss. Media 1 which was diluted in BME/PO buffer with 0.5 g % HSA (MM) was the poorest diluent. The addition of 1 to 2 g % HSA to this media did improve recoveries somewhat (Media 3 and 2).

In direct comparison of stabilizers, the water-based diluents were superior to the salt-based diluents (i.e. Media 5/4 versus Media 7/6).

In the second trial, EMEM/bicarb buffer was substituted for BME/PO_4 in Media 3. Media 5 was the same as in the first run. As seen in Table 19, the water-based media with degraded gelatin and sorbitol (#5) was better than the salt-based Media 3. Since the data were derived from single titrations only, it is more difficult to draw any specific conclusions.

It is planned to conduct two additional drying runs, one slow and one fast using at least two stabilizers to define the conditions needed before drying of the actual vaccine.

Table 17

Safety Tests on Four Junin Virus and Control Fluid Samples

Test		Candidate #1 Material Tested			
		Secondary seed, pool 9 April 82	Secondary seed control fluid 2nd harvest 19 April 82	JV Vaccine 18 June 82	JV Vaccine Control Fluid 2nd harvest 28 June 82
1. Rabbit(3 week - Death/total) Modified 630.16(a)(1)		0/2	0/2	0/2	1/2 Test 1[1] 2/10 Test 2[2]
2 Adult Mice (21 day-Death/total)		0/20	0/20	0/20	0/20
3. Tissue culture Safety Modified 630.16(a)(5-7)	FRhL-2	Passed	Passed	Passed	Passed
	Primary GMK	Passed	Passed	Passed	Passed
	Primary RK	Passed	Passed	Passed	Passed
	MRC-5	CPE Test 1[3]	Passed	CPE Test 1[3]	Passed
		CPE Test 2[4]		CPE Test 2[5]	
4. Bulk Sterility - 610.12		Passed	Thio 6/10Cont.Test 1 Soybean 1/10Cont. Thio 1/10 Test 2 Soybean 1/10 Passed Test 3[6]	Passed	Passed
5. Cultural TB (Lowenstein-Jensen)		Passed	Passed	Passed	Passed
6. PPLO	Cultural	Passed	Passed	Passed	Passed
	DNA Staining	Passed	Passed	Passed	Passed
	Direct FA[7]	Passed	Passed	Passed	Passed

1] Died on day 10.
2] One died on day 6, one on day 18. Nothing remarkable during necropsy.
3] Cells exhibiting granular CPE at day 7, 50-60% peeled off by day 14 but remaining cells were HAd negative.
4] Tubes without Rhesus T-23 antiserum had 60-70% CPE, with antiserum 10-15% CPE at 14 days.
5] Tubes without Rhesus T-23 Antiserum had 50% CPE, with antiserum 5% CPE at 14 days.
6] Secondary seed control fluid, 1st harvest 9 Apr 82.
7] Samples negative for M.hyorhinis , M.arginini and A.laidlawii-a

Table 18

Freeze-drying of Junin Candidate #1
Pass 2 in Seven Media
(Diluted 1:30)
First Run [1]

Media	Frozen/thawed titer (no.) (PFU/ml)	Dried titer (no.)	Moisture Content (%)	Recvry from Froz/Control (%)
Control-Virus pool (calculated 1:30 diln)	3.9×10^2 (3)[2]			
1. MM[3] -116 ml (0.5g% HSA)	4.1×10^2 (2)	2.6×10^1 (2)	0.26	4/2
2. MM-106.4 ml HSA -9.6 ml (2.5 g% HSA)	6.1×10^2 (1)	1.9×10^2 (2)	0.04	31/59
3. MM-112.4 ml MSA-3.6 ml (1.25 g % HSA)	4.1×10^2 (2)	1.3×10^2 (2)	0.13	31/33
4. MM-63.2 ml, HBSS (10x) 4.8ml Degraded gelatin (11%)24 ml Sorbitol (11%) 24 ml	7.2×10^2 (1)[4]	2.4×10^2 (2)	0.12	33/62
5. Distilled H_2O 65.6 ml HSA-2.4 ml (0.5 g % HSA) Degraded gelatin (11%) 24ml Sorbitol (11%) 24 ml	6.3×10^2 (2)	6.3×10^2 (3)	0.06	100/162
6. MM-102.8 ml, HBSS(10x)1.2ml Lactose (22%) 12 ml	3.6×10^2 (2)	1×10^2 (2)	1.06	28/26
7. Distilled H_2O - 101.6 ml HSA - 2.4 ml (0.5 g % HSA) Lactose (22%) 12 ml	5.9×10^2 (2)	2×10^2 (2)	0.06	34/51

1. Drying run was over a period of 4 days - alternating refrigerant between shelves and condenser to maintain product frozen since vacuum was poor. Final drying was achieved between days 3 and 4 with shelves at +25 C to +30°C. Product removed at a temp. of +24 to +25°C.
2. Average of three titers 1.16×10^4 - divided by dilution of 1:30 for virus.
3. MM was BME with PO_4 buffer with 0.5 g % HSA. 4 ml virus added to each media at pH 7.6.
4. One titer of 3.2×10^3 eliminated.

Table 19

Freeze-drying of Junin Candidate #1
Pass 2 in Two Media
(Diluted 1:30)
Second Run[1]

Media	Frozen/thawed titer (no.) (PFU/ml)	Dried titer(no.)	Moisture Content (%)	Recvry from Frozen/Cont (%)
Control-virus Pool (Calculated 1:30 diln.)	1.8×10^4(1)[2]			
3. MM[3] - 112.4 ml HSA-3.6 ml (1.25g % HSA)	9.4×10^4(1)	2.3×10^4(1)	0.2%	24/128
5. Distilled H_2O 65.6 ml HSA - 2.4 ml (0.5g% HSA) Degraded gelatin (11%) 24 ml Sorbitol (11%) 24 ml	1.07×10^5(1)	3.7×10^4(1)	2.8%	35/206

1] Drying run was 24 hours - shelves placed at 25°C when good vacuum achieved - product removed when product reached @ + 19°C (22 hours)
2] Titer was 5.34×10^5 - divided by dilution of 1:30 for virus.
3] MM was EMEM with 0.5 g % HSA. 4 ml virus added to each media at pH 7.6.

Korean Hemorrhagic Fever

1. Diagnostic Reagents Produced

A total of 20 lots of 10 spot FA slides were produced and shipped this year. The 20 lots including 1 normal lot and 19 Hantaan virus infected lots, provided 5900 slides for use. In addition a practice has been established to maintain three lots, approximately 1000 slides, in inventory to meet unforeseen requirements that arise from time to time.

One lot of two pools of rabbit anti Hantaan virus reference antisera prepared last year was aliquoted in 0.2 ml amounts and freeze dried to provide approximately 200 vials for each pool. Both pools titer 1:640 after rehydration. Pool one antisera contains moderate levels of Reovirus FA Antibody (1:160), whereas pool 2 contains barely detectable Reovirus FA Antibody (1:20).

Two reference virus lots were aliquoted and freeze dried. One lot prepared by Dr. Joseph McCormick of the CDC is a large plaque variant of Hantaan virus at Apodemus passage 5, A-549 cell passage 14 and vero cell passage 16. Rehydrated vials titer 1.5 x 10^5 FFU/ml. One hundred and twenty vials of this Lot along with 30 vials of freeze dried Lot 1 pool 2 antisera were shipped to Mr. David Stevens of the American type Culture Collection for distribution by that organization. The second virus lot was prepared by Mr. Orville Brand of USAMRIID. Two hundred seven vials of the material at Apodemus passage 5 A549 cell passage 15 were prepared.

II. Procedure Development

A double staining FA technique utilizing combinations of fluorescein and rhodamine tagged anti-species antiserum conjugates have been evaluated for use in identifying specific fluorescence at low serum dilutions. This procedure appears highly useful for this purpose and may aid in eliminating false positives in screens of large numbers of sera. To achieve this end the Leitz Dialux 20 UV Microscope was fitted with two filter cubes, the H-2 which contains fluorescein exciter filters and the N-2 which holds the corresponding rhodamine exciters. Fluorescein and rhodamine goat anti-rabbit conjugates were then individually titrated in the usual fashion against four units of Lot 1 pool 2 rabbit antiserum. Combinations (mixtures) of the two conjugates were then titrated again, this time switching back and forth from the H-2 and N-2 filter cubes. This allows the same infected cell to be examined by two different conjugates and identical patterns increase the confidence that the observed fluorescence is in fact specific. The procedure worked quite well although it was observed that approximately 1 1/2 times the final concentration of each conjugates is required to yield the same level of fluorescence as when the conjugates were used individually. The rhodamine conjugate (Kirkegaard & Perry Laboratories, affinity purified

a relatively bright red specific fluorescence. Sera invariably titer 2-4 fold higher with this conjugate than with fluorescein conjugates, presumably because of the very low non-specific background.

Tularemia Vaccine Development

I. Introduction

The production methods outlined in Table 20 were used to produce three batches of tularemia vaccine bacteria, strain LVS, in a fermentor. The batches were separately concentrated by Pellicon ultra-filtration; the concentrates were pooled and diluted with an equal volume of sucrose-gel-agar for storage at -30C until lyophilized. Tests have shown that 50 percent of the viable organisms were lost after three months of storage.

Tests done on the bacterial fluids and concentrates at The Salk Institute demonstrate the sterility and identity of these fluids for vaccine use. Dr. H. Eigelsbach has confirmed our test results separately and a copy of his report is attached. Animal tests in guinea pigs and mice indicate the vaccine is similar to previously prepared vaccine.

II. Experimental Vaccine Production of Tularensis for Vaccine.

A flow diagram outlining the production procedure is shown as Table 20.

A. Vaccine seed and growth on solid agar media. (steps 1-3)

The seed bacteria was obtained from lyophilized Pasteurella Tularensis Vaccine, NDRB101, lot 3 produced in this laboratory in 1965 and stored at -10 to -20C. After rehydration the bacterial suspension containing 2×10^9 organisms was streaked on peptone-cysteine-agar (PCA) and incubated for four days at 37C. The transfer and inoculation onto glucose-cysteine-hemin agar (GCHA) was done with inocula from an area on the PCA cultures free of undesirable "gray" colonial growth. After incubation of the GCHA media for 18 hours at 37C a second transfer on this medium was done and further incubation for 18 hours at 37C. The bacterial growth washed from the second GCHA cultures and pooled (120ml) was the inoculum for the first broth culture.

B. First culture in Modified-Casein Partial Hydrolysate (MCPH) Broth. (step 4)

This culture is done in a two-liter bottle fitted with a 10 mm fine glass filter, (sparger) and an airway to vent the sparging air. The sparger is connected to a tank of "breathable air" through an inline filter. Four hundred ml of MCPH broth is placed in the culture flask with 110-120 ml of the bacterial pool from the GCHA cultures. Anti-foam agent (0.1 ml Dow Corning medical AF, 10% solution in water, autoclaved) is added to suppress the formation of foam. The culture is aerated (sparged) at approximately 0.5 liter of air per liter of medium per minute for 18 hours at 37C. At the end of the incubation period the viable titers were 7-12 $\times 10^9$ bacteria per ml and measured 550-700

on a Klett-Summerson spectrophotometer (blue filter).

C. Second Culture in MCPH Broth (step 5)

This culture was done to increase the volume of bacteria for inoculation of the fermentor. This was done in a nine-liter bottle fitted with a 30 mm fine glass filter (sparger). Four liters of MCPH broth containing 0.5 ml of anti-foam was seeded with 500-540 ml of first broth culture fluid. Incubation with sparging (0.5 ml air/min./liter of culture) was for 18 hours at 37C. After incubation the titer and spectrophotometric reading were similar to those obtained in the first broth culture.

D. Fermentor batch culture. (Steps 6,7)

The 50-liter fermentor fitted with a clay filter sparger was charged with 31 liters of medium and five ml of antifoam agent. Four to 4.4 liters of second broth culture seed bacteria were dispensed into the fermentor and the culture was sparged (0.5 liter air/minute/liter of culture fluid) for 18 hours at 34-37C (temperature rises during first six hours of aeration).

After incubation the culture fluid was dispensed into a 75-liter stainless steel pressure tank and stored at 3-5C until concentrated. A sample was obtained to conduct tests for sterility, viable titer, antibiotic sensitivity, colonial morphology and agglutination with known anti-tularensis serum. Table 21 summarizes the test data.

E. Concentration (Steps 8-9)

Four to seven days after harvest, when sterility test demonstrated absence of contamination, the 35-liter batch was concentrated by filtration through a Pellicon ultra-filter system. The final volume of concentrate (retentate in the system) was approximated by weight and later by direct measurement.

The concentrate from each batch was stored at 3-5 C for periods of 7-33 days (see Table 22) until pooled for storage. The first batch concentrate was titrated after 20 and 26 days in the refrigerator. Viable counts were initially 6×10^9 ml, at 20 days, 6.3×10^9; at 26 days 3.9×10^9. The data suggest that the viable count decreases with storage at this temperature.

An aliquot of the diluted pool of concentrates was sent to Dr. H. Eigelsbach for identity tests. A copy of his report is appended here as Table 23. His in vitro test results confirm those done at TSI.

F. Storage at -30 (Steps 10-12)

The storage temperature of -30C was selected based on P. Mazurs' work. (Cited by RM Fry 1966. Freezing and Drying of Bacteria pg. 669-671. In H.T. Meryman (ed.) Crybiology. Academic Press, Inc. New York). Prior to storage the concentrates of three fermentor batches were pooled and diluted with an equal volume of sucrose-gel-agar

saline. The 9.6 liters was dispensed into aliquots and will be stored until lyophilized in final containers.

A test for potency was done in animals on the pooled concentrate using guinea pigs and mice as described in NDBR 101 production protocol. As detailed in Tables 24 and 25, the results are in agreement with those from the earlier vaccine and are within established parameters.

G. Monitor Tests on Vaccine Concentrate Stored at -30 C .

Monitor tests on F.tularensis concentrates stored at -30 C indicate some loss has occurred but the rate of loss appears to diminish with time as shown below:

	(Count)	(Loss/day)
Pre-storage	171×10^9	
7 days	97×10^9	10.5×10^9
94 days	44×10^9	0.6×10^9

III. Conclusion

The fermentation procedure for producing F. tularensis worked well and produces bacteria of a similar quality and quantity as the original shaker flask procedure with less handling. Storage of concentrates requires constant monitoring of the live organisms and is not an optimal procedure as witnessed by the initial losses observed.

Table 20

Flow Diagram

Tularensis Bacterial Growth in a 35 Liter Fermentor

Bacterial Seed

1. "Streak Plates" peptone-cysteine agar
   4 days 37°C
2. 1st "Slant" hemin-agar
   18 hours 37°C
3. 2nd "Slant" hemin-agar
   18 hours 37°C
4. 1st "Broth" culture in MCPH broth 560 ml
   18 hours 37°C - air sparged
5. 2nd "Broth" culture 4.4 L
   18 hours 37°C - air sparged
6. 3rd "Broth" culture - fermentor 35 L
   18 hours 37°C - air sparged
7. Harvest - store 5°C
   2 - 5 days
8. Concentrate - Pellicon filtration to 1.5 - 2.0 Liters
9. Storage 5°C
10. Pool Concentrates (Concentrates of 3, 35 liter runs)
11. Dilute Pool 1:1 (Sucrose-gel-agar)
12. Store -30°C

Table 21

Testing On Three Fermentor Batches (35 liters) of
Francisella tularensis Bacteria

Strain LVS
(Pasteurella Tularensis Vaccine NDBR101, lot 3)

Process and Tests	Batch 1	Batch 2	Batch 3
Viable titer/ml x 10^9	6	8.5	11.2
Sterility: thioglycollate	S[1]	S	S
Soy-bean casein hydrolysate	S	S	S
Blood agar base[2]	No growth	No growth	No growth
Gram stain	Negative	Negative	Negative
Growth on peptone-cysteine agar[3]	S	S	S
Agglutination[4]	Positive	Positive	Positive
Antibiotic sensitivity:[5]			
Streptomycin 2 mgm	17mm	15mm	15mm
Streptomycin 10 mgm	23mm	20mm	21mm
Chloramphenicol 5mgm	23mm	15mm	23mm
Chloramphenicol 30mgm	37mm	32mm	35mm

1] S=test satisfactory
2] Blood agar base medium does not have cysteine required for tuleremia growth
3] Colonial morphology was typical tularemia growth. Only a few "gray" colonies were observed
4] Anti-SCHU-4 rabbit serum strongly agglutinated the bacteria
5] Expected diameters of inhibition are:

Streptomycin:	2 mgm.	15-17 mm
	10 mgm.	20-23 mm
Chloramphenicol	5 mgm.	15-17 mm
	30 mgm.	30-37 mm

Table 22

Concentration and Storage of Tularemia Batches
Produced by Fermentation

Process and Tests	Batch 1	2	3
Volume Fermentor Batch (liter)	35	35	35
Volume Concentrated Batch (liter)	1.2	1.2	2.4
Viable Titer Fermentor Batch/mlX10^9 [1]	6	8.5	11
Viable Titer Concentrate mlx10^9 [2]	130	930	340

Viable titer pooled concentrates (Diluted 1:1 with SGA) pre-freezing: 19.2×10^{10}

Viable titer pooled concentrate (Diluted 1:1 with SGA) 4 days - 30C: 9.7×10^{10}

[1] Batch #1 concentrate stored 3-5C for33 days before pooling.
Batch #2 Concentrate stored 3-5C for 16 days before pooling.
Batch #3 Concentrate stored 3-5C for 7 days before pooling.
Titers shown were done on the day of harvest.

[2] Viable titers immediately after concentration was done.

Table 23

DEPARTMENT OF THE ARMY
U.S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES
FORT DETRICK, FREDERICK, MARYLAND 21701

IN REPLY REFER TO

Identity Testing of Tularensis Vaccine:
Conc. 9-9-82

SGRD-UIB-A

29 September 1982

Dr. George R. French
The Salk Institute
Government Services Division
P.O. Box 250
Swiftwater, PA 18370

Dear Dr. French:

Two samples of Live Tularensis Vaccine labeled, "Tularensis Vaccine; Conc. 9-9-82; 5 ml; Ref. 172-20" and packed in dry ice were received on 14 September 1982. Both vials were in good condition and were surrounded with ample remaining dry ice to preserve the frozen condition. Both vials were transferred to a Revco freezer and held at -70°C until evaluated.

On 17 September 1982 one vial, designated vial #1, was immersed in water at 37°C and quickly thawed with agitation. Samples were used for Gram staining, agglutination with specific antiserum, purity, antibiotic sensitivity, colonial morphology, and viable count. All tests were performed in accordance with the procedures developed for evaluation of Live Tularensis Vaccine. Results are as follows:

Vial #1

Gram Stain	Gram negative. Morphology resistant with Francisella tularensis. No other microorganisms observed.
Slide Agglutination with Specific Antiserum	Strongly positive
Purity (Thioglycolate Broth; 10 tubes each inoculated with 0.25 ml)	No growth of any potential contaminant within 7 days at 37°C.
Antibiotic Sensitivity (Disc Method)	Streptomycin +
	Tetracycline +
	Kanamycin +
	Chloramphenicol +
	Penicillin -
	Ampicillin -

Colonial Morphology on PCA	Essentially no colony-type variation. Homogeneous colony-type consistent with F. tularensis strain LVS.
Viable Count (thawed vaccine passed through 22 gauge needle 5 times to ensure suspension of individual microorganisms before dilution and plating).	1.2 X 10"/ml

Vial #2

(thawed and tested on 21 September 1982)

All results for Vial #3 are the same as reported for Vial #1 except for a slight difference in viable count.

Viable Count for Vial #2 1.3 X 10"/ml

Cordially,

HENRY T. EIGELSBACH, Ph.D.
Senior Scientist (Ret)

Table 24

Guinea Pig Virulence and Immunogenicity
of *F. tularensis* vaccine
(Dilute fluid vaccine)

Route of Inoculation	No. Viable org./dose	Reaction Days 1-15	Post Challenge[1] (days 16-31) Survival	Percent Protection
		(no./total)	(no./total)	
Subcutaneous	10^4	Survival 10/10	3/10	30
	10^8	Survival 10/10	8/10	80
Multiple Puncture	6×10^5 [2]	Typical skin rx[3]	7/10	70

1 Challenge dose was given subcutaneously and contained 10^3 virulent SCHU-4 strain *F. tularensis*.

2 One drop (0.06 ml) of a dilution of vaccine containing 10^7 org./ml with 60 skin punctures using a 26 G hypodermic needle.

3 On days 1-8 the skin at the site of inoculation was inflamed (erythema) and during the second week a pustule formed which resolved by sloughing with scar formation.

Table 25

Mouse Virulence and Immunogenicity

Vaccine Dose[1]	Survival Days 1-15	Virulence	Survival Post Challenge[2] Days 16-31	Protection
(No. Viable Org.)	(No./total)	(%)	(No./total)	(%)
10^7	17/20	15	17/20	100
10^6	17/20	15	17/20	100
10^5	19/20	5	19/20	100
10^4	19/20	5	19/20	100
10^3	19/20	5	19/20	100
10^2	20/20	0	20/20	100
Diluent Control	20/20	-	0/20	0

1]Vaccine inoculated subcutaneously, 0.2 ml (inoculum contained number of organisms indicated).

2]Challenge dose was given subcutaneously and contained 10^3 virulent SCHU-4 strain F. tularensis.

Dengue 1 Vaccine

I. Introduction

Dengue 1 vaccine candidate seeds and Parent viruses were transferred from USAMRIID to TSI-GSD this year. Two passages of Strain 45AZ5 were made in certified FRhL-2 cells to prepare a Master Seed and a Production Seed (P4 in FRhL-2). One passage of strain TP 79-56 was made in FRhL-2 to prepare a Production Seed (P29 in FRhL-2). After testing and pooling, each production seed was passed once in certified FRhL-2 cells to prepare vaccine virus. The harvests were frozen at -70°C in ten separate pools while testing was done. Strain 45AZ5 was thawed, pooled and freeze-dried in two runs. A monkey viremia study was done in addition to the required safety tests. Testing that remains to be done on both strains includes monkey neurovirulence and reverse transcriptase. Strain TP 79-56 vaccine is still frozen in ten pools.

II. Process Studies

A. Dengue 1, Strain 45AZ5

The passage history of this strain is shown in Table 26.

A master seed virus was prepared in FRhL-2 cells from a seed labelled "45AZ5, 2nd 103 pass from day 6, 1st pass-day 9, 5 June 81'. Control fluid was of the same date. Harvest was on day 7 when 1-2+ CPE occurred. The harvest virus (EMEM + 2% V/V HSA) was supplemented with HSA to 8 percent V/V and adjusted to pH 7.6 before freezing at -70°C. Control cells were normal for 14 days and were negative for hemadsorption to guinea red blood cells. All harvests were sterile.

Production seed was prepared from the Master Seed at a dilution of 1:100. It was harvested after 6 days in 9 pools, the HSA content raised to 8 percent, pH 7.6 and frozen.

After preliminary testing, the production seed (P4 in FRhL-2) was pooled and dispensed into convenient aliquots for testing and further use. It was stored at -70°C. In addition to safety test samples, 480 ml was frozen in bulk and 231 vials of 2.2 ml each were dispensed.

A 1:100 dilution of the production seed was used to inoculate 345 x 150 cm^2 cultures of FRhL-2, Lot 21 with 5 ml each. After 90 minutes adsorption, the seed was removed, cultures washed with HBSS and EMEM with 2 percent HSA (MM) added. Sixty control cultures were inoculated with Passage 4 control fluid and treated identically. Virus and control fluids were harvested after 5 days at 35°C. Virus was proportionately harvested into 10 pools,

supplemented with HSA to 8 percent (V/V) and adjusted to pH 7.6. Samples were removed and pools were frozen at -70°C. The vaccine pools were thawed in cold water, pooled and freeze-dried in two runs. Pre-drying titer was 1.5×10^4 PFU/ml. The freeze drier was not operating properly during these runs due to a persistant leak which was later found and repaired. Post-drying titers were 2.8×10^4 (Run 1) and 3.8×10^5 (Run 2).

B. Dengue 1, Strain TP 79-56

The passage history of this strain is given as Table 27.

A production seed was prepared from a Master Seed obtained from USAMRIID labelled, TP79-56, 50/50 Master Seed, 35° Pool, 25 Oct (79), D8. Control fluid was obtained of the same date. The viral fluids (EMEM + 2 percent V/V HSA) were harvested in ten pools, HSA content raised to 8 percent V/V and pH adjusted to 7.6 before freezing at -70°C. Pools were combined and aliquoted after titers, plaque size and ts were determined. Control cultures were normal for 14 days and were negative to hemadsorption with guinea pig red blood cells. A total of 1.2 liters of production seed, 106, 1.2 ml and 145, 2.2 ml aliquots were frozen in bulk in addition to safety test samples.

A 1:500 dilution of the production seed was used to inoculate 345 x 150 cm^2 cultures of FRhL-2, Lot 21 with 5 ml each. Procedures and harvest after 5 days were the same as for 45AZ5 (A. above). A mock pool of the vaccine had a plaque titer of 2.5×10^5 PFU/ml. All preparations were free of bacterial contamination. This vaccine is being maintained at -70° C in ten pools.

C. Testing

1. Safety tests

The status of safety testing for 45AZ-5 and TP79-56 strains of Dengue 1 are summarized in Table 28. Monkey neurovirulence and reverse transcriptase testing has not been done for either strain. The suckling mouse test that failed for TP79-56 vaccine virus is being further evaluated by subpassage and specific neutralization. Other tests such as stability checking were interrupted by reconditioning of the building and will be picked up later.

2. Titrations, ts and plaque-size

The available data on titer, ts and plaque size are summarized in Table 29 for 45AZ-5 strain and in Table 30 for TP79-56 strain. Again this work was temporarily halted for building reconditioning.

In relation to the temperature sensitivity and plaque size data, both candidate viruses occasionally plaque under the agar overlay and replicate in liquid overlay at 39.3 °C. This occurs with master seed, production seed and bulk vaccine. Plaque size for 45AZ-5 is uniformly small ranging up to 1 mm with an average of less than 0.5 mm. TP79-56 is small but less uniform than 45AZ-5. They average about 1 mm or less, but a fair proportion in all three passage levels approach 2 mm diameter. Both virus strains are clearly distinguishable from Parent 2. However, Parent 1 is not much different from TP79-56. The average plaque size is 1.5 mm with an occasional plaque exceeding 2.0 mm.

There are clear differences between both candidate viruses and both parents in plaque titer and yield ratios at two temperatures (35°C/39.3°C) Parent 1 is intermediate but still different. The plaque titer ratio for Parent 2 is $\sim$ 10, Parent 1 $\sim$ 30, while 45AZ-5 and TP79-56 are always >500 and often don't plaque at 39.3°C. Liquid overlay average yields at the two temperatures show the same thing, Table 31.

3. Monkey study on Dengue Type 1

Tables 32 and 33 summarize the viremia, HI, FA and $PRNT_{50}$ titers in 12 monkeys that received either Parent 2, or strains 45AZ-5 or TP79-56 viruses. Outgrowths of both viruses from these studies for ts, plaque size and titrations in suckling mice on the TP79-56 and Parent 1 need to be accomplished yet.

Table 26

Passage History of Dengue 1, Strain 45AZ5

Virus Designation	FRhL-2 (DBS 103) Passage Number	Seed Virus Dilution [1]	Date Harvested-day	PFU/ml (LLC-MK2)	By
1. Seed	2	-	06/--/81-9	1×10^4	USAMRIID
2. Master Seed	3	1:100	06/02/82-7	2.1×10^4	TSI
3. Production Seed	4	1:100	06/17/82-6	6.4×10^4	TSI
4. Vaccine	5	1:100	07/26/82-5	1.5×10^5 [2]	TSI

[1] 5 ml of diluted virus added to each 150 cm^2 flask and adsorbed 90 minutes/35.5°C.

[2] Pre-drying titer. Dried in two runs. Post-drying titer Run 1, 2.8×10^4, Run 2, 3.8×10^4.

Table 27

Passage History of Dengue 1, Strain TP79-56

Virus Designation	FRhL-2 (DBS 103) Passage Number	Seed Virus Dilution [1]	Date Harvested-day	PFU/ml	By
				(LLC-MK2)	
1. Master Seed	28	-	10/25/79-8	2.3×10^4	USAMRIID
2. Production Seed	29	1:500	06/01/82-6	2.9×10^5	TSI
3. Vaccine	30	1:500	08/09/82-5	$1.7-2.5 \times 10^5$	TSI

[1] 5 ml of diluted virus added to each 150 cm^2 flask and adsorbed 90 minutes/35.5°C.

Table 28

Testing of Dengue 1 Candidate
Vaccine Virus

	Test	45AZ-5 Prod. Seed		45AZ-5 Vaccine (Lot 1-82)		TP79-56 Prod. Seed		TP79-56 Vaccine (Lot 2-82)	
		Control Fluid	Virus	Control Fluid	Virus	Control Fluid	Virus	Control Fluid	Virus
1.	In process sterility	S[1]	S	S	S	S	S	S	S
2.	In process TB	S	S	S	S	S	S	S	S
3.	In process PPLO	S	S	S	S	S	S	S	S
4.	Bulk sterility	S	S	S	S	S	S	S	S
5.	Two week hold of control cells and hemadsorption	S	S	S	S	S	S	S	S
6.	Rabbit safety	S	S	S	S	S	S	S	S
7.	Suckling mouse safety	S	S	S	S	S	S	S	S[2]
8	Guinea pig safety	S	S	S	S	S	S	S	S
9.	Adult mouse safety	S	S	S	S	S	S	S	S
10.	Tissue culture safety-FRhL-2	S	S	S	S	S	S	S	S
11.	Tissue culture safety-PRK	S	S	S	S	S	S	S	S
12.	Tissue culture safety-MRC-5	S	S	S	S	S	S	S	S
13.	Tissue culture safety-PGMK	S	S	S	S	S	S	S	S

Testing of Dengue 1 - continued

Test		45AZ-5 Prod. Seed		45AZ-5 Vaccine (Lot 1-82)		TP79-56 Prod. Seed		TP79-56 Vaccine (Lot 2-82)	
		Control Fluid	Virus	Control Fluid	Virus	Control Fluid	Virus	Control Fluid	Virus
14.	Reverse transcriptase	-	ND[1]	-	-	-	ND	-	-
15.	Monkey neurovirulence	ND	ND	-	-	ND	ND	-	-
16.	Breakthrough neut. test	-	S	-	S	-	S	-	S
17	Final container Sterility Run 1	-	-	-	S	-	-	-	ND
	Run 2	-	-	-	S	-	-	-	ND
18.	Final container Moisture Run 1	-	-	-	0.25%	-	-	-	ND
	Run 2	-	-	-	0.29%	-	-	-	ND
19.	Final container General safety Run 1	-	-	-	S	-	-	-	ND
	Run 2	-	-	-	S	-	-	-	ND
20.	Final container Potency Run 1	-	-	-	ND	-	-	-	ND
	Run 2	-	-	-	ND	-	-	-	ND
21.	Final container Stability, 4°C reconstituted Run 1	-	-	-	ND	-	-	-	ND
	Run 2	-	-	-	ND	-	-	-	ND
22.	Final container stability, 4°C Run 1	-	-	-	ND	-	-	-	ND
	Run 2	-	-	-	ND	-	-	-	ND

Testing of Dengue 1-continued

Test	45AZ-5 Prod. Seed		45AZ-5 Vaccine (Lot 1-82)		TP79-56 Prod. Seed		TP79-56 Vaccine (Lot 2-82)	
	Control Fluid	Virus	Control Fluid	Virus	Control Fluid	Virus	Control Fluid	Virus
23. Final container identity								
Run 1	-	-	-	ND	-	-	-	ND
Run 2	-	-	-	ND	-	-	-	ND
24. Final container stability -20°C								
Run 1	-	-	-	ND	-	-	-	ND
Run 2	-	-	-	ND	-	-	-	ND

1/ S = Test was acceptable
ND = Not done
2/ Test failed = animals to be subpassed (7/20 died on 1st test, 5/20 in 2nd test)

Table 29

Preparation and Testing of Dengue 1, Strain 45AZ5

Virus Designation	FRhL-2 (DBS 103) Passage No.	Seed Virus dilution	Date Harvested/ Day	35°C Titer (PFU/ml)[1]	35°C Plaque Size/Ave. (mm)	39.3°C Titer (PFU/ml)[1]	39.3/35°C Ratio
1. Seed	2	-	6/--/83 - 9	1×10^4			
2. Master Seed	3	1:100	6/02/82 - 7	3.6×10^4	0.5-1.0/~0.5	$<1.0 \times 10^1$	<0.00028
3. Mock Production Seed[2]	4	1:100	6/17/82 - 6	1.5×10^5	0.5-1.0/~0.5	$<1.0 \times 10^1$	<0.000067
Actual Production Seed				2.1×10^5	0.5-1.0/~0.5	$<1.0 \times 10^1$	<0.000048
4. Mock Vaccine[2]	5	1:100	7/26/82 - 5	1.5×10^5	0.5-1.0/~0.5	$<1.0 \times 10^1$	<0.000067
Actual Vaccine				5.6×10^4	0.5-1.0/~0.5	2.0×10^2	0.0036
5. Freeze-Dried Vaccine			Run 1	2.8×10^4			
			Run 2	3.8×10^4			
6. Parent 2 Test 1 (P8)	-	-	6/02/81 - 6	6.5×10^6	0.5-2.5/2.0 (D14) 1.0-4.0/2.7 (D28)	6.0×10^5	0.092
Test 2 (P8)	-	-	6/02/81 - 6	1.2×10^7	0.5-2.5/2.0		
Test 3 (P8)				5.0×10^5		6.3×10^4	0.126

1/ LLC-MK2
2/ Equal aliquots from each pool combined.

Table 30

Preparation and Testing of Dengue 1, Strain TP79-56

Virus Designation	FRhL-2 (DBS 103) Passage No.	Seed Virus Dilution	Date Harvested/ Day	35°C Titer (PFU/ml)[1]	35°C Plaque Size/Av. (mm)	39.3°C Titer (PFU/ml)[1]	39.3/35°C Ratio
1. Master Seed (35°C Pool)	28	—	10/25/79 - 8	1.9×10^4	0.5-2.0/<1.0[1]	$<1.0 \times 10$	<0.00053
2. Mock Production Seed[2]				4.0×10^5	0.5-2.0/<1.0[1]	$<1.0 \times 10$	<0.000025
Actual Production Seed	29	1:500	6/01/82 - 6	6.6×10^5	0.5-2.0/<1.0		
3. Mock Vaccine[2]				2.5×10^5	0.5.0-2.0/<1.0		
Actual Vaccine	30	1:500	8/09/82 - 5	5×10^4	ND[3]	9.3×10^2	0.019
4. Parent 1 Test 1 (P8)				1.2×10^7	0.5-2.0/∿ 1.0 (D14) 1.0-4.0/1.5 (D28)	7.5×10^5	0.058
Test 2 (P8)			1/22/81	1.6×10^7	0.5-2.0/∿ 1.2		
Test 3 (P8)				2.5×10^5		1.4×10^4	0.057

1] LLC-MK2
2] Equal aliquots from each pool combined.
3] ND = Not Done

Table 31

Liquid Overlay Peak Yields

At Two Temperatures

Virus	Average Peak Yield 35°C	39.3°C	Ratio 35°C/39.3°C
	(PFU/ml)		
45AZ5	5.6×10^4	2.0×10^2	280
Parent 2	5.0×10^5	6.3×10^4	8
TP79-56	5.0×10^4	9.3×10^2	54
Parent 1	2.5×10^5	1.4×10^4	18

Table 32
Dengue Virus Type 1 - Monkey Study

Virus/Dose	Monk. #	Dil.	Viremia - Day Post Infection Total PFU Observed/Total T-25* 2	3	4	5	6	7	8	9	10	11	12	Ave. T°C	Av.Wt. (kg)
TP-79-56	540C	1:3	0/4	0/6	0/6	0/6	2/6	0/6	3/6	0/4	8/4	4/4	0/2	38.9±0.2	8.6±0.2
9.5×10^3	906B	1:3	1/4	0/6	0/6	5/6	0/6	5/6	0/6	8/4	18/4	8/4	2/2	38.9±0.2	9.8±0.4
	962B	1:3	0/4	0/6	0/6	0/6	0/6	7/6	5/6	0/4	6/4	4/4	0/2	38.6±0.2	7.7±0.0
	958A	1:3	0/4	0/6	0/6	0/6	2/6	1/6	12/6	7/4	2/4	1/4	1/2	39.1±0.4	8.6±0.1
45AZ5	532C	1:3	0/4	0/6	0/6	0/6	0/6	1/6	2/6	1/4	2/4	0/4	0/2	38.7±0.3	7.6±0.7
1.8×10^4	B7481	1:3	0/4	0/6	2/6	0/6	6/6	12/6	5/8	2/4	0/4	0/4	4/2	39.1±0.2	6.0±0.2
	755B	1:3	2/4	0/6	3/6	0/6	0/6	2/6	2/6	0/4	0/4	9/4	0/2	38.7±0.2	6.3±0.2
	5693	1:3	13/4	0/6	0/6	2/6	4/6	1/6	9/6	0/4	0/4	0/4	0/2	38.5±0.4	7.6±0.5
Parent 2	354C	1:3	0/4	0/6	0/6	44/6	185/6	264/6	8/6	7/6	0/4	0/4	1/2	38.3±0.2	8.0±0.6
3.3×10^4	454C	1:3	0/4	7/6	19/6	125/6	263/6	158/6	108/6	15/6	0/4	0/4	0/2	38.8±0.2	7.0±0.5
	468C	1:3	1/4	1/6	8/6	12/6	24/6	39/6	29/6	11/6	0/4	0/4	0/2	38.8±0.2	7.4±0.8
	928B	1:3	0/4	0/6	6/6	3/6	47/6	263/6	48/6	7/6	0/4	0/4	0/2	39.2±0.3	9.9±0.2

* 0.2ml of 1:3 diluted serum/T-25

Table 33

Dengue Virus Type 1 - Monkey Study

Virus/dose	Monk.#	Day 0			Day 14		Day 21		Day 28		Day 60	
		HI	FA	$PRNT_{50}$	HI	FA	HI	FA	HI	FA	FA	$PRNT^{80}/50$
TP-79-56 9.5×10^3	540C	<10	<10	<10	<10	<10	40	80	160	160	160	40/320
	906B	<10	<10	<10	<10	<10	80	160	80	320	160	300/≥1280
	962B	<10	<10	<10	<10	<10	20	80	20	80	40	40/330
	958A	<10	<10	<10	<10	<10	40	80	160	160	80	40/330
45AZ5 1.8×10^4	532C	<10	<10	<10	<10	<10	40	80	40	80	40	15/130
	B7481	<10	<10	<10	10	10	40	160	40	160	40	6/50
	755B	<10	<10	<10	<10	10	40	40	40	80	20	5/50
	5693	<10	<10	<10	<10	<10	10	80	40	160	80	160/1280
PARENT 2 3.3×10^4	354C	<10	<10	<10	80	80	160	320	320	640	160	80/640
	454C	<10	<10	<10	40	80	80	320	320	640	320	160/1280
	468C	<10	<10	<10	20	80	80	160	80	160	80	160/1280
	928B	<10	<10	<10	40	40	160	320	160	320	160	40/320

Diagnostic Antiserum Preparation

I. Introduction

Anti-dengue virus mouse ascitic fluid and anti-bovine virus diarrhea rabbit sera were prepared. These stock reagents are stored at -65C and will be tested for use.

II. Experimental

A. Anti-Dengue 1 Mouse Ascitic Fluid (MAF)

The anti-Dengue virus mouse ascitic fluid was prepared by a technique described by Brandt, et al (Amer. J. Trop. Med. Hyg. 16: 339). A fresh stock of virus was prepared in suckling mice by i.c. inoculation with Dengue virus, type I (Hawaii strain 80374-7, pass 17-TSI suckling mouse brain virus). The infected brain tissue in 20% suspension (PBS - 2% normal rabbit serum) had a titer of $10^{-7.33}$/ml, MICLD .

The following immunization schedule for the adult mice (3-4 months) was used:

Day 0: 9 mice were each inoculated with virus (20% brain tissue) 0.4 ml s.c. and 0.5 ml Freund's complete adjuvant, i.p.

Day 4: Inoculations repeated

Day 25: Each mouse inoculated i.p., 0.5 ml, with the virus emulsified in adjuvant, 1:1.

Day 27: Repeat emulsified virus inoculation

Day 30: Repeat emulsified virus inoculation

Day 39: Obtained 65 ml of ascitic fluid from 6 mice by centesis (2-20 ml/mouse).

The ascitic fluid was clarified by centrifugation and stored at -65C. It maintained neutralization of Dengue 45-AZ5 and TP79-56 strains of Dengue 1 in tissue culture through a dilution of 1:128.

B. Anti-bovine diarrhea virus antiserum

The anti-bovine diarrhea virus antiserum was prepared by inoculation of each of three rabbits, 0.1 ml i.v. and 0.5 ml i.m. (alhydrogel adjuvant added). Two rabbits received a live virus obtained from Ames Laboratories (grown in bovine kidney/goat serum) and one animal received a virus from NIH (grown in bovine kidney/fetal bovine serum).

After two inoculations on days 0 and 14, sera (3-5 ml) were obtained on day 35 for testing and a third inoculation was done. On day 43 the rabbits were bled from the heart and the sera processed.

Aliquots of the serum from each rabbit are stored at -65 C. It is intended for use in spot-slide evaluations.

Drug Screening Program

I Introduction

Plaque reduction tests were completed with RVF virus, five drugs, Pichinde virus, seven drugs, and JBE (Oct-541) virus, twenty drugs.

Five drugs were tested by yield inhibition tests against Pichinde virus.

Peak yield harvest times were done against VEE, Pichinde and RVF viruses.

Seventeen drugs were tested in the drug screening program by plaque reduction for their effect on RVF, VEE, JBE and Pichinde viruses during the last quarter of 1982. Results were forwarded to USAMRIID.

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